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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of :  
Yoshio UMEZAWA et al. : Attn: BOX PCT  
Serial No. NEW : Docket No. 2002\_0426A  
Filed March 26, 2002 :

PROBE FOR PROTEIN-PROTEIN INTERACTION  
ANALYSIS, AND METHOD OF USING IT FOR  
ANALYSIS OF PROTEIN-PROTEIN INTERACTION  
[Corresponding to PCT/JP00/09348  
Filed December 27, 2000]

**PRELIMINARY AMENDMENT**

Assistant Commissioner for Patents,  
Washington, DC 20231

Sir:

Prior to calculating the filing fee, please amend the above-identified application as follows:

**IN THE SPECIFICATION**

**Page 1, immediately after the title, please insert:**

This application is a 371 of PCT/JP00/09348 filed December 27, 2000.

**Please replace the paragraph beginning on page 7, line 11 with the following rewritten paragraph:**

Fig. 2 shows the N-terminal polypeptide structure of EGFP in the Example of the present invention, for which the 124th to 129th amino acid residues have been replaced.

ATTACHMENT D

**Please replace the paragraph beginning on page 9, line 7 with the following rewritten paragraph:**

Fig. 9 shows the time-dependent protein splicing increase in the Example of this invention.

**Please replace the paragraph beginning on page 12, line 2 with the following rewritten paragraph:**

Specifically, intein derived from yeast VMA and Ssp DnaE intein derived from cyanobacterium are preferable. In yeast VMA, the nascent translation product, 120-kDa VMA1, catalyzes protein splicing to give a 70-kDa H<sup>+</sup>-ATPase sub-unit and a 50-kDa site-specific endonuclease (VDE, or PI-SceI). This VDE is preferably used as the intein sites (2a, 2b) in the probes for protein-protein interaction analysis (1a, 1b). For the Ssp DnaE derived from cyanobacterium, the DNA sequence of the strain PCC6803 has been determined (the N-terminal has 123 amino acid residues and the C-terminal has 36 amino acid residues); further, Ssp DnaE intein is a natural split intein, and is known that the ligation of N- and C-extein occurs (Wu, H., Hu, Z., Liu, X., Xu, M. Q., *Proc. Natl. Acad. Sci. USA* 1998, 95, 9226-9231). Therefore Ssp DnaE intein is easy to handle, and preferred as the intein sites (2a, 2b) in the present probes for protein-protein interaction analysis (1a, 1b).

#### **IN THE CLAIMS**

**Please amend the claims as follows:**

4. **(Amended)** The probe for protein-protein interaction analysis of claim 2, wherein the intein is an endonuclease derived from yeast VMA.

5. **(Amended)** The probe for protein-protein interaction analysis of claim 2, wherein the intein is DnaE derived from cyanobacterium.

6. **(Amended)** The probe for protein-protein interaction analysis of claim 2, wherein the labeled protein is a fluorescent protein.

8. **(Amended)** The probe for protein-protein interaction analysis of claim 2, wherein the labeled protein is a luminescent enzyme.

10. **(Amended)** A method for analyzing protein-protein interaction, comprising:  
making a protein linked with probe a as described in claim 2 and a protein linked with probe b as described in claim 2 coexist in a system;  
and detecting the signal emitted by the labeled protein.

11. **(Amended)** The method of claim 10, wherein a polynucleotide is introduced into a eucaryotic cell, the polynucleotide expressing the protein linked with probe a and the protein link with probe b, thereby making the protein linked with probe a and the protein linked with probe b coexist in the cell.

**REMARKS**

The specification has been revised to reflect the national stage status.

Minor editorial corrections have been effected to the specification which are self-explanatory.

In addition, the multiple dependencies of the claims have been removed to eliminate improper multiple dependencies and to reduce the PTO filing fee.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached pages are captioned "**Version with markings to show changes made.**"

Favorable action on the merits is solicited.

Respectfully submitted,

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March 26, 2002

**Version with Markings to  
Show Changes Made****10/089040****IC10 Rec'd PCT/PTO 26 MAR 2002****DESCRIPTION**

**PROBE FOR PROTEIN-PROTEIN INTERACTION ANALYSIS, AND METHOD OF  
USING IT FOR ANALYSIS OF PROTEIN-PROTEIN INTERACTION**

*This application is a 371 of PCT/JP00/09348 filed December 27, 2000.*

**TECHNICAL FIELD**

The invention of this application relates to a probe for protein-protein interaction analysis, and to a method of using such probe for the analysis of protein-protein interactions. More precisely, the invention relates to a probe for protein-protein interaction analysis and to a method of using it for analysis of protein-protein interactions, which enables accurate and simple analysis of protein-protein interactions in all living cells.

**BACKGROUND ART**

It is well known that protein-protein interactions play key roles in structural and functional organization of living cells.

Many unsolved problems currently studied in molecular biology and biochemistry, such as for gene transcription mechanism and intracellular information transmission are related to protein-protein interactions.

Some of the problems in the art of molecular biology and biochemistry have been gradually solved by the development of protein library screening techniques such as a two-hybrid

splicing; and (IV) shows linkage of labeled protein. (1a) indicates probe a for protein-protein interaction analysis; (1b) indicates probe b for protein-protein interaction analysis; (2a) indicates the N-terminal polypeptide of an intein; (2b) indicates the C-terminal polypeptide of the intein; and (3) indicates a labeled protein. Further, (3a) indicates the N-terminal polypeptide of the labeled protein; and (3b) indicates the C-terminal polypeptide of the labeled protein. (4a) indicates protein (or a protein site) A; and (4b) indicates protein (or protein site) B.

Fig. 2 shows the N-terminal polypeptide structure of EGFP in the Example of the present invention, for which the <sup>124th</sup> ~~1124th~~ <sup>129th</sup> to ~~1129th~~ amino acid residues have been replaced.

Fig. 3 shows the data of SDS-PAGE analysis of proteins expressed in *E. coli* transformed with pGEX-NVC. Here, lanes (a) to (c) are Coomassie Blue-stained SDS-PAGE; lane (a) indicates the protein molecular mass standard (Novagen) with their molecular masses (kDa); lane (b) indicates the crude extract before purification by GST-affinity column; and lane (c) indicates the sample purified by GST-affinity column. Lanes (d) and (e) show Western blotting analysis of crude lysate using antibodies specific to VDE (d) and to GFP (e).

Fig. 4 shows the structure of a plasmid (probe <1> for protein-protein interaction analysis) constructed in the Example of the present invention. Here, the restriction

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intracellular ribosome extein site (IRES); the cDNAs of pLucAll, pLucN and pLucC are inserted into pCDNA3.1 (+); "stop" and "start" indicate translation termination and initiation codons, respectively.

Fig. 8 shows the emission intensity of LucN alone, LucC alone and LucAll in the Example of this invention.

Fig. 9 shows the <sup>time</sup>insulin-dependent protein splicing increase in the Example of this invention.

Fig. 10 shows the influence of amino acid mutation on protein splicing in the Example of this invention.

Fig. 11 shows the insulin concentration dependency of Lum-F emission intensity in the Example of this invention.

**BEST MODE FOR CARRYING OUT THE INVENTION**

The probe for protein-protein interaction analysis of the present invention is based on the principle that the interaction of two proteins each linked to the probes induces splicing thereby regenerating the labeled protein split and attached to the two probes, to emit a signal.

Protein splicing is a process wherein the internal protein segment (intein) is excised from a translated protein. In this process, the excision of inteins is accompanied by the ligation of flanking sequences (exteins) (Gimble, F. S., Sci. Biol. 1998, 5, R251-256).

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excision.

Specifically, intein derived from yeast VMA and Ssp DnaE intein derived from cyanobacterium are preferable. In yeast VMA, the nascent translation product, 120-kDa VMA1, catalyzes protein splicing to give a 70-kDa H<sup>+</sup>-ATPase sub-unit and a 50-kDa site-specific endonuclease (VDE, or PI-SceI). This VDE is preferably used as the intein sites (2a, 2b) in the probes for protein-protein interaction analysis (1a, 1b). For the Ssp DnaE derived from cyanobacterium, the DNA sequence of the strain PCC6803 has been determined (the N-terminal has 123 amino acid residues and the C-terminal has 36 amino acid residues); further, Ssp DnaE intein is a natural split intein, and is known that the ligation of N- and C-extein occurs (Wu, H., Hu, Z., Liu, X., Xu, M., Q., X., Q., Proc. Natl. Acad. Sci. USA 1998, 95, 9226-9231). Therefore Ssp DnaE intein is easy to handle, and preferred as the intein sites (2a, 2b) in the present probes for protein-protein interaction analysis (1a, 1b).

Of the various inteins mentioned above, the cyanobacterium-derived Ssp DnaE intein is most preferable for enabling highly sensitive detection of protein-protein interactions in mammal cells. It is needless to say, that any other known or novel inteins may also be employed in the invention.

For effective intein splicing (III) with the probes for protein-protein interaction analysis (1a, 1b) of the present invention, probes 1a and 1b must be correctly folded so that





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protein.

8. <sup>(Amended)</sup> The probe for protein-protein interaction analysis of ~~claims 2 to 5~~, wherein the labeled protein is a luminescent enzyme.

9. The probe for protein-protein interaction analysis of claim 8, wherein the luminescent enzyme is a luciferase.

10. <sup>(Amended)</sup> A method for analyzing protein-protein interaction, comprising:

making a protein linked with probe a as described in ~~claims 2 to 9~~ and a protein linked with probe b as described in ~~claims 2 to 9~~ coexist in a system;

and detecting the signal emitted by the labeled protein.

11. <sup>(Amended)</sup> <sup>Claim 10,</sup> The method of ~~protein-protein interaction analysis~~ of ~~claim 10~~, wherein a polynucleotide that ~~expresses the probe~~ of ~~any one of claims 1 to 9~~ is introduced into a eucaryotic cell, thereby making the ~~probe a-linked protein and the probe b-linked~~ protein coexist in the cell.

the protein linked with probe a  
and the protein linked with probe b

the polynucleotide expressing the  
protein linked with probe a and the  
protein link with probe b, thereby